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(54) Title: SWINE PNEUMONIA VACCINE AND METHOD FOR THE PREPARATION THEREOF (57) Abstract The invention provides vaccine components and compositions useful for vaccinating pigs against <i>Mycoplasma hyopneumoniae</i> and secondary pathogenic infections.		

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SWINE PNEUMONIA VACCINE AND METHOD
FOR THE PREPARATION THEREOF

Field of the Invention

5 This invention relates to vaccines against infection by pathogens of the genus Mycoplasma and, more particularly, to bacterins useful as prophylactic agents to inhibit infection by Mycoplasma, particularly Mycoplasma hyopneumoniae.

10

Background of the Invention

Mycoplasma hyopneumoniae is a ubiquitous swine respiratory pathogen causing mycoplasmal pneumonia in swine (MPS). MPS occurs worldwide and is considered to
15 be one of the most common and economically important diseases affecting swine. Transmission of M. hyopneumoniae apparently occurs through direct contact with infected respiratory-tract secretions or aerosol droplets. It has been identified as the primary pathogen
20 in the enzootic pneumonia complex. This disease has been estimated to cost in excess of \$100 million annually, primarily due to its adverse effects on affected animals.

M. hyopneumoniae infection results in a chronic
25 infection causing a lingering nonproductive cough and stunted growth, resulting in overt disease symptoms at the "growing and finishing stage", i.e., 6 weeks of age or older, in pigs. Severe lung damage to the infected

animal, and/or death may occur at 4 to 6 months of age. Because most naturally occurring cases of MPS are mixed infections involving mycoplasmas, bacteria, viruses and parasites, death of the animal is usually due to
5 secondary bacterial, viral or other pathogenic infections.

In the twenty-five years since the discovery of Mycoplasmas as the causative agent for enzootic pneumonia in 1965, researchers have pursued a variety of means to
10 control and treat this disease and develop a safe and effective vaccine against Mycoplasma hyopneumoniae. Protection against infection induced by vaccine candidates is preferably measured by reduction in the number of lung lobes with lesions, reduction in the
15 percent of lung lobes with lesions, reduction in mean lung lesion scores, reduction in microscopic lesion scores, and reduction in severity of M. hyopneumoniae infection as measured by a fluorescent antibody test.

Several experimental vaccines have produced
20 less than optimal results. Studies have shown that strong immunity developed during the course of experimentally-induced MPS. However, when pneumonic lung tissue is administered as an antigen, animals become even more susceptible to challenge. Formalinized cultures of
25 M. hyopneumoniae have been shown not to be protective.

[See, also, e.g., C. A. Brandly et al, eds., "Advances in Veterinary Science and Comparative Medicine", Vol. 17,

Academic Press, NY (1973) and references cited therein;
R. F. W. Goodwin et al, A. Hyg. Camb., 67:465 (1969)].
Extracts of M. hyopneumoniae in ether or sodium
dodecylsulfate (SDS), and a repeat freeze-thaw product
5 have been shown to give significant protection against
lung homogenate challenge [K. M. Lam et al, Am. J. Vet.
Res., 32:1737 (1971)].

Sonicated cells of M. hyopneumoniae in Tween 80
and paraffin oil resulted in good indirect
10 hemagglutination (IHA) titers in the blood, colostrum and
milk when injected into the mammary gland [S. Durisic et
al, Acta Vet. (Beograd), 25(4):189-194 (1975)]. Whole
cell preparations and cell-free supernatants have been
shown to be only partially protective [R. F. Ross et al,
15 Am. J. Vet. Res., 45(10):1899 (1984)]. Plasma membranes
from sonically disrupted cells, adjuvanted with $Al_2(OH)_3$,
or agarose, gave good passive protection [M. Kobisch et
al, Ann. Inst. Pasteur/Immunol., 138:693-705 (1987)].

A live, avirulent LKR strain of M.
20 hyopneumoniae was also shown to give good protection in
pigs against challenge with a virulent M. hyopneumoniae
strain [L. C. Lloyd et al, Abstract 4593 in Bacteriology
and Bacterial Diseases, from Australian Vet. J., 66(1):9-
12 (1989)].

25 Infection by M. hyopneumoniae is presently
controlled, in part, with various classes of antibiotics,
such as tetracycline, lincomycin, and tiamulin. However,

antibiotics are of limited therapeutic value because they do not prevent the establishment of an infection, and lung lesions may develop after treatment ends. The presence of secondary pathogens also makes selection of the appropriate antibiotic difficult [R. Landon, Topics in Vet. Med., 1(1):14-21 (1990)].

Other approaches which are presently in use to reduce the impact of chronic respiratory diseases caused by M. hyopneumoniae, are minimal disease systems such as the Swedish and British systems and farrowing of older sows. Minimization of stress, optimal management conditions and all-in, all-out systems of production are recommended.

There remains a need in the art for an effective vaccine against M. hyopneumoniae, which would confer protection against M. hyopneumoniae challenge and also significantly reduce the morbidity and mortality from secondary respiratory pathogens, such as Pasteurella multocida.

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Summary of the Invention

In one aspect the invention relates to a composition useful for vaccinating pigs against Mycoplasma hyopneumoniae. This composition comprises inactivated M. hyopneumoniae organisms in a pharmaceutically acceptable medium, the organisms being present in an amount sufficient to induce an

immunological response in pigs protective against challenge by M. hyopneumoniae.

In a further aspect, the present invention provides a vaccine composition comprising the inactivated
5 M. hyopneumoniae composition above in combination with additional vaccine components, including one or more components capable of inducing protection against atrophic rhinitis following infection by P. multocida, and other agents infectious for swine. Such vaccines may
10 include immunogenic amounts of one or more of the following vaccine components: a stable, soluble, cell-free toxoid of P. multocida, a whole Pasteurella multocida bacterin with cell-bound toxoid, a B. bronchiseptica bacterin or an Erysipelothrix
15 rhusiopathiae bacterin-extract, an Actinobacillus pleuropneumoniae bacterin, a Haemophilus parasuis bacterin, and/or viral vaccine components, such as from a Pseudorabies virus. Other conventional vaccine components may also be added to the vaccine compositions
20 of this invention.

As still another aspect, the invention provides a method for producing the vaccine components described above. The M. hyopneumoniae vaccine component is
25 prepared by a series of steps including pretreatment of the culture medium with ion exchange resins, such as the Amber-lites resin, increasing the dissolved oxygen content of the inoculated culture to between 20 and 40%,

and inactivating the culture with a selected inactivating agent.

Yet a further aspect of this invention includes a method for increasing the resistance of swine to M. hyopneumoniae infection comprising administering an effective amount of the vaccine compositions of the present invention to swine.

Still another aspect of this invention includes a method for protecting swine against M. hyopneumoniae infection and other pathogenic infections comprising sequentially or simultaneously administering to an animal an effective amount of the M. hyopneumoniae vaccine and one or more vaccines containing an additional antigen, e.g., from pathogens as identified above.

Other aspects and advantages of the present invention are described in the following detailed description of preferred embodiments of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides vaccine components, vaccine formulations, and methods for their preparation and use in pigs as an aid in the prevention of infection by M. hyopneumoniae. The vaccine components and vaccines of this invention confer protection against M. hyopneumoniae challenge with a wild-type strain as well as other known virulent strains. Embodiments of the

invention are also capable of significantly reducing the morbidity and mortality from secondary respiratory pathogens, such as Pasteurella multocida.

Mycoplasma hyopneumoniae strains useful in preparing the vaccines of the present invention can be isolated from swine infected with wild-type or other known strains causing mycoplasmal pneumonia in swine. Other known strains of M. hyopneumoniae, both virulent and non-virulent, may be useful in the compositions of this invention. Useful strains may be obtained from commercial or academic collections, such as the American Type Culture Collection in Rockville, Maryland, U.S.A. A particularly preferred strain of M. hyopneumoniae for use in embodiments of this invention is identified as strain P-5722-3, ATCC Accession No. 55052, deposited on May 30, 1990 pursuant to the accessibility rules required by the United States Patent and Trademark Office.

A vaccine component according to this invention may be prepared by inoculating a medium capable of supporting the growth of Mycoplasma, particularly M. hyopneumoniae, with a selected M. hyopneumoniae seed stock. A selected culture medium may include media known to those of skill in the art to propagate Mycoplasma, such as described in Freundt, cited above, and other prior art. A particularly desirable medium formulation for culturing of the M. hyopneumoniae is described in Example 1 below and includes PPLO broth, yeast extract,

heat inactivated serum, cysteine hydrochloride, dextrose, an antibiotic to inhibit bacterial growth, and an optional agent to indicate growth and to avoid excess pH change, e.g., phenol red.

5 Preferably, the selected medium is pretreated with an anion exchange resin, Amberlite (chloride form) resin. This step is believed to enhance growth of the organism by removing inhibitory agents. Other conventional methods, such as gel filtration, may also
10 prove useful in this pretreatment step. This pretreatment step is preferably applied to the broth before addition of the other medium components and prior to inoculation. Exposure to the ion exchange resin may be maintained between one to four hours at a rate of
15 approximately 500 grams/10 liters of broth.

 Once pretreated, the medium is inoculated with a suspension of the selected M. hyopneumoniae strain, at preferably a 5-20% inoculation. The culture is thereafter incubated at a temperature of between
20 approximately 30°C to 40°C. A more preferred temperature range is 35°C to 38°C, with 37°C being particularly preferred.

 In a particularly preferred embodiment, the dissolved oxygen content of the culture is raised to a
25 level of between 20% to 40% of saturation. A preferred oxygen content of the culture during the incubation is approximately 25%. Cultures containing a dissolved O₂

content of greater than 20%, produce higher titers of the organism. Increasing the dissolved oxygen content is accomplished by conventional means in the art, such as by aeration with sterile air and agitation. This particular
5 step in the culturing of the vaccine component is believed to provide enhanced titers of M. hyopneumoniae.

The pH of the culture is maintained at neutral to slightly alkaline pH. Desirably the pH of the culture is maintained between 6.2 to 7.9. A more preferable pH
10 range is 7.0 ± 0.5 . The desired pH may be maintained by the addition of sterile NaOH, when necessary.

Cultures are incubated for 36 to 168 hours. More preferably the incubation period is between 36 and 96 hours, until a minimum titer of 1×10^8 color changing
15 units (CCU) in liquid titration medium [A.W. Rodwell and R.H. Whitcomb (1983) in Methods in Mycoplasmaology, Vol. 1, Chapter 14; Shmuel Razin and Joseph G. Tulley, Eds.] is achieved. More preferably the titer is at least 5×10^8 CCU/ml, and may be up to 5×10^{10} CCU/ml as determined
20 by CCU or an ELISA.

At the end of the culture period, the organisms are inactivated by the addition of a known inactivating agent. One such preferred inactivating agent is binary ethyleneimine (BEI). Other inactivating agents may
25 include, for example, formaldehyde or glutaraldehyde. The inactivating agent is added in an approximate amount of 4.0 mM. Once the inactivating agent is added, the

culture is incubated again with agitation for at least 24 hours.

The inactivating agent may be removed or neutralized by conventional means, and the culture
5 incubated again for at least 24 hours to complete neutralization of the inactivating agent. Once inactivated, the vaccine component may be formulated into a vaccine for administration to animals.

The present invention also contemplates vaccine
10 components for use in combination with the M. hyopneumoniae vaccine component described above. Vaccine components, including inactivated bacterins or purified toxoids, from one or more pathogens, such as Pasteurella multocida, Streptococcus suis, Actinobacillus
15 pleuropneumoniae, Haemophilus parasuis, Bordetella bronchiseptica, Salmonella choleraesuis and ascaris larva, may also be employed in conjunction with the vaccine components described above in combination
vaccines or in therapeutic methods involving sequential
20 or simultaneous co-administration. Such a combination vaccine is prepared by mixing an immunogenic amount of inactivated M. hyopneumoniae as described above and an immunogenic amount of a bacterin or toxoid of another
pathogen with suitable adjuvants and physiologic vehicles
25 for injection into mammals. A co-administration therapy may employ one or more of the above antigens formulated into individual vaccines. The M. hyopneumoniae vaccine

and additional selected vaccine may be administered via the same routes of administration, using different sites for administration. Administration may be sequential or simultaneous.

5 Preferred embodiments of such combination vaccines or vaccine co-administration therapies include immunogenic amounts of one or more of the following vaccine components: a stable, soluble, cell-free toxoid of P. multocida, a whole Pasteurella multocida bacterin
10 with cell-bound toxoid, whole cell P. multocida, type A and type D, a B. bronchiseptica bacterin or an Erysipelothrix rhusiopathiae bacterin-extract. Pending United States patent application SN07/537454 is incorporated by reference to provide disclosure of
15 methods for preparing preferred P. multocida vaccine and combination vaccine components. Vaccines described in that application may also be combined with the M. hyopneumoniae vaccine component of the present invention to form combination vaccines.

20 Additional vaccine combinations with the M. hyopneumoniae component of this invention may include the antigens Actinobacillus pleuropneumoniae, Haemophilus parasuis, and Pseudorabies virus. These additional components may be useful in a vaccine formulation or
25 therapy for weaned pigs, preferably for administration as early as 3 to 6 weeks of age.

Based on at least one challenge, it appears that a vaccine co-administration with one vaccine containing the M. hyopneumoniae vaccine component and a second vaccine containing a P. multocida vaccine component referred to above evidenced no immunosuppressive effects by the M. hyopneumoniae vaccine on the animals as determined by seroconversion responses.

Vaccines of the invention may be prepared as pharmaceutical compositions containing an effective immunogenic amount of the inactivated organism, as active ingredients in a nontoxic and sterile pharmaceutically acceptable carrier. Such a vaccine may comprise the inactivated vaccine component described above mixed with optional preservatives and emulsifiers.

Alternatively or additionally, the inactivated M. hyopneumoniae may be admixed or adsorbed with a conventional adjuvant. The adjuvant is used as a non-specific irritant to attract leukocytes or enhance an immune response. Such adjuvants include, among others, Amphigen, mineral oil and lecithin, aluminum hydroxide, muramyl dipeptide, and saponins such as Quil A.

A preferred embodiment of the vaccine of the invention contains an aqueous suspension or solution containing the inactivated P-5722-3 strain of M. hyopneumoniae, preferably buffered at physiological pH, in a form ready for injection.

It is preferred that the vaccine of the invention, when in a pharmaceutical preparation, be present in unit dosage forms. For purposes of this invention, a desirable immunogenic amount of inactivated
5 organism, when administered as the sole active ingredient is between 5×10^8 (CCU) and 5×10^9 (CCU). The vaccine of the invention is preferably administered in two 2 ml doses, each dose containing the desired titer.

Preferably the doses are administered two weeks apart.

10 Primary immunization of piglets should be initiated at approximately one week of age with a booster dose 2 weeks later. For primary immunization of pregnant swine, two doses are recommended approximately four weeks apart with the last dose administered two weeks before
15 farrowing. A booster dose is recommended prior to each subsequent farrowing. Semi-annual vaccination is recommended for boars.

It is preferred that the vaccine of the invention, when in a pharmaceutical preparation, be
20 present in unit dosage forms. Dosage forms preferably contain about 2 ml. For purposes of this invention, an immunogenic amount of the inactivated M. hyopneumoniae, when administered as the sole active ingredient is between about 5×10^8 to 5×10^9 CCU per dose. In a vaccine
25 composition containing additional antigenic components, the same immunogenic amount or a reduced amount of M. hyopneumoniae may be employed.

Other appropriate therapeutically effective doses can be determined readily by those of skill in the art based on the above immunogenic amounts, the condition being treated and the physiological characteristics of the animal. In the presence of additional active agents, these unit dosages can be readily adjusted by those of skill in the art. A desirable dosage regimen involves administration of one or two doses of desired vaccine composition, where the antigenic content of each fraction is desirably as stated above. Of course, the administration can be repeated at suitable intervals if necessary or desirable.

The mode of administration of the vaccines of the invention may be any suitable route which delivers the vaccine to the host. However, the vaccine is preferably administered by intramuscular injection. Other modes of administration may also be employed, where desired, such as subcutaneously, intradermally, intraperitoneally or intranasally.

The following Examples of the invention are illustrative only and not intended to be limiting.

EXAMPLE 1

Propagation and Culture of *M. hyopneumoniae*

M. hyopneumoniae strain P-5722-3 was furnished courtesy of Dr. Charles Armstrong, Purdue University, and deposited with the American Type Culture Collection under

Accession No. 55052. This strain has the immunochemical and biochemical characteristics of being mannose positive, arginine negative, and urease negative. The strain is positive for growth inhibition with anti-M. hyopneumoniae antiserum and positive by direct
5 fluorescent antibody test with anti-hyopneumoniae fluorescein-conjugated antibody. This strain was propagated as described below.

A culture medium was prepared according to the
10 following procedure. An 83% PPLO broth, without crystal violet [Difco Laboratories, Detroit, Michigan] was conditioned by treating the broth with an anion exchange resin [Amberlite, Sigma IRA400-chloride form] for one to four hours, at the rate of 500 grams of resin for every
15 ten liters of broth.

Yeast extract was prepared by adding five hundred grams of active yeast granules to three liters of distilled or deionized water, stirred at room
temperature. After thorough mixing, the suspension was
20 stirred for an additional 15-45 minutes after which 16.2 ml of 10 N NaOH was added, dropwise. The slurry was then autoclaved for 15-45 minutes at 121°C. The supernatant was decanted into a container and clarified by either centrifugation or microfiltration. To the clarified
25 supernatant, 1 N HCl was added at a rate of 2 ml per 100 ml extract. The extract was stirred for at least fifteen minutes at room temperature and then clarified as

described above. The clarified extract was sterilized by autoclaving as described above or by microfiltration.

To the pretreated broth the following media components were added: 0.01% thallium acetate; 0.005%
5 ampicillin; 0.0125% cysteine hydrochloride; 6.25% yeast extract, 1% dextrose; 10% swine serum (Gibco) heat inactivated; and, optionally, 0.0026% phenol red. The pH of the culture medium was adjusted to $\text{pH } 7.5 \pm 0.2$ and filter sterilized.

10 To initiate a production serial, frozen M. hyopneumoniae master seed was thawed and a 5-20% suspension inoculated into 100-3000 ml of the culture medium described above. The culture was incubated at 30°C to 39°C for 36 to 168 hours. Following satisfactory
15 growth, the culture was transferred into a seeding container with fresh medium, using a 5-20% inoculum. This culture was incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 36 to 96 hours.

Production cultures of M. hyopneumoniae are
20 grown in fermentors, incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 36 to 96 hours following inoculation. The dissolved oxygen content of the culture is maintained at between 20-40% by aeration with sterile air and agitation. Sterile antifoam may be used to control foam.

25 At the end of the growth period, the pH of the culture was raised to 7.6 ± 0.2 and the pH maintained in this range for about one hour. To inactivate the

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organism, a filter-sterilized aqueous solution of 2-bromoethylaminehydrobromide (BEA) was added to a final concentration of approximately 4.0 mM. BEA is converted to the inactivating agent binary ethyleneimine (BEI) at the increased pH of the culture. The culture was incubated at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with constant agitation for at least 24 hours.

After the 24 hour incubation, a filter sterilized aqueous solution of sodium thiosulfate, a standard neutralizing agent, was added to a final concentration of approximately 4 mM to neutralize excess BEI. The culture was incubated for an additional 24 hours at $37^{\circ}\text{C} \pm 1^{\circ}\text{C}$ to complete inactivation.

15

EXAMPLE 2

Preparation of a Vaccine

Following inactivation of the vaccine component of Example 1, a vaccine was formulated by adding to the inactivated M. hyopneumoniae several conventional vaccine components. Sufficient inactivated M. hyopneumoniae was combined with phosphate buffered saline diluent to obtain a minimum antigen concentration of 5×10^8 CCU and a maximum of 5×10^9 CCU of M. hyopneumoniae per 2 ml dose. Sterile 10% merthiolate and 10% ethylenediamine tetra acetic acid (EDTA, disodium or tetrasodium salt) solutions were added as preservatives. Sterile mineral oil [Drakeol] containing 5% to 40% by weight of lecithin

(Central Soya) was added as an adjuvant. The final concentration of between 0.7% to 3.2% Tween 80 and 0.3% to 1.8% Span was added as an emulsifier. Selected parabens (methyl p-hydroxybenzoate, propyl p-hydroxybenzoate, butyl p-hydroxybenzoate) may be added as additional preservatives for the oil and emulsifiers.

EXAMPLE 3

Vaccine Challenge Experiments

Two separate vaccination-challenge experiments were conducted to evaluate the protective capabilities of an inactivated, adjuvanted M. hyopneumoniae vaccine in swine.

Thirty-three, crossbred, six and one-half week old pigs were obtained from a closed, respiratory disease-free herd and allocated to one nonvaccinated challenged control group (Group 1), two vaccinated challenge groups (Groups 2 and 3), and one nonvaccinated nonchallenged control group (Group 4).

Animals in Group 2 received the vaccine described in Example 1 except that the M. hyopneumoniae culture was inactivated with 0.3% formalin instead of BEI; and animals in Group 3 received the vaccine described in Example 1, in both cases administered in 2 ml doses intramuscularly on day "0" and day 14. Both vaccines contained inactivated whole cells adjuvanted the same way, differing only with respect to the inactivating

agent used. Pigs in Group 4 received 6 ml doses of incomplete Freund adjuvanted Friis mycoplasma broth (placebo) via the same route.

Pneumonia was induced by intratracheal inoculation of pigs [Bentley, O.E. and Farrington, D.O. 1980. Am. J. Vet. Res. 41:1870] seven days after the second dose of vaccine with a single 10 ml dose of crude lung homogenate containing M. hyopneumoniae strain 232 [derived from strain 11]. Pigs in Group 4 were given 10 ml. Friis mycoplasma broth.

Necropsy was performed approximately 3 weeks post-challenge. Criteria utilized for determination of efficacy of vaccines for prophylaxis of M. hyopneumoniae disease were (a) severity of clinical signs; (b) macroscopic lesions of pneumonia; (c) microscopic lesions typical of the disease; and (d) infection of lung tissue determined by immunofluorescence [Amaneu, W. et al. Proceedings, IPVS Congress, Copenhagen, Denmark. p. 223 (1980)].

As shown in Tables 1 and 2 below, pigs in Groups 1 and 2 tended to have slightly higher coughing scores than pigs in Groups 3 and 4. All pigs in Group 1 (positive control) and most pigs in Groups 2 and 3 (vaccinated) had lesions. None of the pigs in Group 4 had lesions. Numbers of lobes with lesions were substantially less in Group 3 pigs than pigs in Groups 1 and 2. Most importantly, mean percent of lungs with

pneumonia and mean lung lesion scores were significantly less in vaccinated Group 3 than in Group 1. Severity of pneumonia in Group 2 was not significantly different from Group 1 (Table 1).

5 Most pigs in all groups had some microscopic lesions. Severity of microscopic lesions were significantly less in Groups 2, 3 and 4 than in positive Group 1.

10 Evaluation of lungs by immunofluorescence revealed that 7 out of 8 pigs in Group 1 were positive for the disease. Only four in ten pigs and three in nine pigs were FA positive in vaccinated groups 2 and 3, respectively.

15 All pigs in Groups 2 and 3 had developed high complement fixing (CF) antibody titers to M. hyopneumoniae by the time they were challenged. A more rapid development of CF antibody positive status was observed with Group 2 than with Group 3.

20 In summary, the vaccine administered in Group 3 pigs provided relatively strong protection against intratracheal challenge with M. hyopneumoniae lung homogenate. Protection was evidenced by the reduced number of lobes with lesions, reduced percentage of lungs with lesions, reduced mean lung lesion scores, reduced
25 microscopic lesion scores, and reduced number of pigs FA positive.

21

Table 1

	<u>Groups</u>	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
	No. of pigs	8	10	9	6
	Vaccine	-	XMHP4	XMHP5	Placebo
5	Mean Coughing Score \pm S.D.	1.11 \pm 0.11	1.13 \pm 0.15	1.05 \pm 0.10	1.01 \pm 0.02
	<u>Gross Lesions</u>				
10	No. with lesions	8	7	6	0
15	No. lobes positive	35/56	23/70	12/63	0/42
20	Mean % lung with lesions \pm S. D.	7.93 \pm 4.5	6.80 \pm 7.73	1.9 \pm 3.39	0
	<u>Microscopic lesions</u>				
25	No. with lesions	8	7	8	4
30	Mean lesion scores \pm S.D.	2.81 \pm 0.40	2.0 \pm 0.84	1.72 \pm 0.68	1.29 \pm 0.25
	<u>Immunofluorescence</u>				
	No. positive	7	4	3	0
35	Avg. score	0.63	0.28	0.18	0
40	CF Antibody Titers pre-challenge	<4	2521	474	<4

In the second experiment, the efficacy of the inactivated vaccine of Example 1 was confirmed in piglets (conventionally raised), vaccinated at 1 and 3 weeks of age. Experimental design and results are summarized in Table 2.

Table 2

Study II Groups		A	B
No. of pigs		38	29
Dosage/Route		2 ml IM-vaccine	2ml IM-placebo
Challenge		Intranasal	Intranasal
No. pigs	NL1042	25	23
challenged with	ISU232	13	6
% Mean lesion*	NL1042	4.98	27.91
scores	ISU232	1.82	19.24
*Method of Goodwin and Whittlestone			

EXAMPLE 4

Preparing Pasteurella Multocida Toxoid for a
Combination Vaccine

An additional vaccine component for a combination vaccine of this invention may include the following P. multocida toxoid.

A. Culturing the P. multocida

P. multocida type D (strain 8) [Dr. Ross Cowart, University of Illinois, Urbana, Illinois] is subcultured in a modified chemically defined synthetic medium for one day. The medium is described by Herriott et al, J. Bact., 101:513-516 (1970).

The pH of the assembled medium is adjusted to 7.3 ± 0.2 with sterile NaOH. Cells from this culture are transferred to fresh synthetic medium and this culture, when grown, is combined with a cryopreservative and stored at -70°C . Production cultures are grown to harvest during incubation at approximately $36^{\circ} \pm 1^{\circ}\text{C}$ for between 3 and 24 hours following inoculation. The dissolved oxygen content of the culture is maintained by aeration with sterile air and by agitation. Sterile antifoam solution is used to control foam. The pH of the culture is maintained at 7.3 ± 0.2 .

At the end of the growth cycle, P. multocida cultures are examined and cell density is determined by absorbance at 650 nm. Agitation is then decreased, and aeration and pH control are discontinued.

B. Pre-detoxification treatment

Following growth of the organism, sterile merthiolate is added to the culture in an amount less than or equal to 0.01 percent weight per volume. Culture fluids may be aseptically transferred through closed connections to a sterile closed container. The container is connected through closed fittings to an apparatus used to physically lyse cells and release cellular contents, e.g., a "GAULIN" model 15M laboratory homogenizer.

Bacterial cells in the culture fluid are lysed by continuous passage through the pressure chamber of the homogenizer. This subjects the cells to an immediate pressure drop from between an initial pressure of between 2000 and 5000 psi to ambient pressure of 15 psi. The lysed cells are aseptically deposited into another closed container.

The lysate is clarified by sequential steps of centrifugation and/or microporous filtration. Clarified solutions may be concentrated before or after filter sterilization. Ethylenediaminetetraacetic acid (EDTA), in an amount up to a final concentration of 5 mM, and glycerol, in an amount up to a final concentration of 1.0% (vol/vol), are added before concentrating and filter-sterilizing, to prevent aggregation of the concentrated proteins.

C. Detoxification

Detoxification is achieved by the following process. Sterile 5 N NaOH is slowly and aseptically added to sterile toxin to increase the pH from its initial level of a pH of approximately 7.0 to a pH of approximately 10.55 ± 0.10 . The pH is maintained at this level for approximately 7 hours. Thereafter the pH is adjusted to 7.0 ± 0.2 by slowly and aseptically adding sterile 4 N HCl. Fluids are held at this pH for 1 hour. Agitation and temperature are maintained at a constant level throughout the process.

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Preferably this detoxification process is performed a total of 3 times within 25 hours or until detoxification is complete. The toxoid is then stored at 2° to 7°C until combined with other components and assembled into vaccine compositions.

Aliquots are taken each time the pH is approximately 7.0, e.g., at the starting pH and whenever the pH is adjusted to approximately 7. Residual toxicity of each aliquot is measured and expressed in mouse LD₅₀'s per mL. A preparation with an initial value of nearly 2,500 LD₅₀'s per mL is usually completely detoxified approximately 25 hours after the pH is first adjusted to 10.55, without appreciable decrease in assayable antigen content.

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EXAMPLE 5

Preparing a P. Multocida Bacterin-Toxoid VaccineComponent

A combination vaccine of this invention may include a bacterin-toxoid of P. multocida in which the toxoid has been stabilized within the bacterial cell.

A culture of P. multocida, type D, strain 4677, is grown in the following medium: Tryptic Soy Broth without Dextrose (Difco) 30 g; Yeast extract (Difco) 5 g; Dextrose 4 g; Deionized water to 1 liter; pH of approximately 7; sterilized by autoclaving at 121°C.

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The culture is aerated with agitation to maintain the dissolved oxygen concentration at approximately 35% of saturation. The temperature is maintained at 37°C, and the pH at 7 by the addition of 10N NaOH solution as needed. Towards the end of exponential growth, aeration is discontinued and the culture is inactivated by the addition of formaldehyde solution (USP) to a final concentration of 0.5% v/v. The culture is then held at 37°C for four days. Other inactivating agents, such as beta-propiolactone, glutaraldehyde, and binary ethyleneamine can be used in place of formaldehyde.

A sample is withdrawn to test whether inactivation is complete by administering the sample to guinea pigs. Guinea pigs should be alive and healthy at 7 days after subcutaneous injection with 4 ml volumes of the culture. At this point the toxin within the cells is completely converted to toxoid, which is safe, very stable and capable of inducing the production of neutralizing antitoxins upon injection into animals.

The inactivated culture is centrifuged. The sedimented bacteria are dispensed in sufficient supernatant fluid to make a suspension with an OD (optical density at 625 nm, as determined in a Spectronic 20 spectrophotometer) of 4.2. The suspension is then adsorbed with Al(OH)₃ gel, 25% v/v, thimerosal (0.01% w/v) is added as a preservative, and the pH is adjusted to 6.5 ± 0.2.

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EXAMPLE 6

Co-administration Experiments with Two Vaccines

To determine if conventional swine vaccines containing Bordetella bronchiseptica, Erysipelothrix rhusiopathiae, and P. multocida whole cell vaccines, type A and D, would adversely be affected by the vaccine containing the inactivated M. hyopneumoniae component of Example 1 a study was conducted. Both vaccines were simultaneously administered by the same route at different sites.

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The M. hyopneumoniae vaccine referred to above evidenced no immunosuppressive effects or other adverse interference on the animals' response to the other vaccine as measured by seroconversion responses.

Alternatively, the M. hyopneumoniae bacterin of this invention may be employed in vaccine compositions with such other vaccine components.

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Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. For example, use of other appropriate strains of M. hyopneumoniae, or strains for combination vaccines, and vaccine components, such as adjuvants, preservatives and the like, may be selected by one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A vaccine component comprising inactivated *M. hyopneumoniae* at a dosage of at least 5×10^8 CCU, said component capable of inducing an immunological response in vaccinated swine against *M. hyopneumoniae*.
2. The vaccine component according to claim 1 produced by pretreating a culture medium capable of sustaining *M. hyopneumoniae* by anion exchange resin; inoculating said pretreated medium with *M. hyopneumoniae*, increasing the dissolved oxygen content of said culture to between 20 to 40% of saturation, culturing the *M. hyopneumoniae* to a titer of at least 1×10^8 CCU, and inactivating the culture by the addition of a selected inactivating agent.
3. The component according to Claim 2 wherein the inactivating agent is binary ethyleneimine.
4. The component according to Claim 2 comprising a titer of 5×10^8 to 5×10^9 color changing units.

5. The component according to Claim 2 wherein said culturing step comprises growing the culture of *M. hyopneumoniae* at a temperature of between 30° to 40°C at neutral to slightly alkaline pH.

6. The component according to Claim 1 wherein the *M. hyopneumoniae* is strain P-5722-3, ATCC Accession No. 55052.

7. A vaccine capable of inducing immunity to *M. hyopneumoniae* in a mammal without serious side effects comprising a vaccinal amount of the component of Claim 1 and an adjuvant to elicit an immunoprotective response in a porcine animal, the component having immunogenic activity in at least an amount sufficient to protect the animal against challenge by *M. hyopneumoniae*.

8. The vaccine according to Claim 7 wherein the component comprises the *M. hyopneumoniae* strain P-5722-3, ATCC Accession No. 55052.

9. The vaccine according to Claim 7 wherein the component comprises the inactivated *M. hyopneumoniae* at a titer of between 5×10^8 to 5×10^{10} color changing units.

10. The vaccine according to Claim 7 wherein said adjuvant is selected from the group consisting of: lecithin and mineral oil, saponins, aluminum hydroxide.

11. A method for the preparation of a vaccine for protecting mammals against *M. hyopneumoniae* which comprises pretreating a culture medium capable of sustaining *M. hyopneumoniae* by anion exchange resin inoculating said medium with *M. hyopneumoniae*, increasing the dissolved oxygen content of said culture to between 20 to 40% of saturation, culturing the *M. hyopneumoniae* to a titer of at least 1×10^8 CCU, and inactivating the culture by the addition of a selected inactivating agent.

12. The method according to Claim 11 wherein the inactivating agent is binary ethyleneimine.

13. The method according to Claim 11 wherein said titer is between 5×10^8 to 5×10^{10} color changing units.

14. The method according to Claim 11 wherein said culturing step comprises growing the culture of *M. hyopneumoniae* at a temperature of between 30° to 40°C at neutral to slightly alkaline pH.

15. The method according to Claim 11 wherein the *M. hyopneumoniae* is strain P-5722-3, ATCC Accession No. 55052.

16. A method for vaccinating a pig against *M. hyopneumoniae* comprising inoculating said pig with a vaccinal amount of the vaccine of Claim 7.

17. A vaccine composition comprising an inactivated *M. hyopneumoniae* vaccine component and an immunogenic amount of one or more additional antigens.

18. The composition according to claim 17 wherein said additional antigens are selected from the group consisting of a *Pasteurella multocida* bacterin with a cell-bound toxoid, a *Bordetella bronchiseptica* bacterin, an *Erysipelothrix rhusiopathiae* antigen extract, a soluble free-toxoid of *Pasteurella multocida* type D, and inactivated whole cells of *P. multocida* type A or D, cultures of *Actinobacillus plueroneumoniae*, *Haemophilus parasuis*, and *Pseudorabies virus*.

19. A vaccine composition according to claim 17 consisting of inactivated *M. hyopneumoniae*, inactivated *Bordetella bronchiseptica*, inactivated *P. multocida* type A, inactivated *P. multocida* type D, *P. multocida* free toxoid, and *Erysipelothrix rhusiopathiae* antigen extract.

20. A vaccine composition according to claim 17 consisting of inactivated *M. hyopneumoniae*, and vaccine components of *Actinobacillus pleuroneumoniae*, *Haemophilus parasuis*, and *Pseudorabies virus*.

21. A method for vaccinating a pig against *M. hyopneumoniae* and secondary bacterial infection comprising inoculating said pig with a vaccinal amount of the vaccine of Claim 17.

22. A method for vaccinating a pig against *M. hyopneumoniae* and secondary bacterial infection comprising inoculating said pig with a vaccinal amount of the vaccine of Claim 18.


23. A method for vaccinating a pig against *M. hyopneumoniae* and secondary bacterial infection comprising inoculating said pig with a vaccinal amount of the vaccine of Claim 19.

24. A method for protecting swine against *M. hyopneumoniae* infection and other pathogenic infections comprising sequentially or simultaneously administering to an animal an effective amount of the vaccine of Claim 7 and one or more vaccines containing an additional antigen.

25. The method according to claim 24 wherein said additional antigen is selected from the group consisting of a *Pasteurella multocida* bacterin with a cell-bound toxoid, a *Bordetella bronchiseptica* bacterin, an *Erysipelothrix rhusiopathiae* antigen extract, a soluble free-toxoid of *Pasteurella multocida*, and inactivated whole cells of *P. multocida* type A or D, cultures of *Actinobacillus plueroneumoniae*, *Haemophilus parasuis*, and *Pseudorabies* virus.

INTERNATIONAL SEARCH REPORT

International Application No. CT/US91/03689

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A61K 39/02, 39/00; C07K 3/00, 13/00, U.S.Cl.: 424/92, 88; 530/413, 416;		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S.	424/92, 88; 530/413, 416	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched ⁸		
Online search conducted on APS, CA and BIOSIS		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ⁹	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
Y	Am. J. Vet. Res., Volume 45, No. 10. issued October 1984. Ross et al.. "Characteristics of protective activity of <u>Mycoplasma hyopneumoniae</u> vaccine," pages 1899-1905, see summary.	1-16
Y	A. Hyg. Camb., Volume 67, issued 1969. Goodwin et al., "Some experiments relating to artificial immunity in enzootic pneumonia of pigs," pages 465-475, see summary.	1-16
Y	US,A, 4,894,332 (Schaller et al.) 16 January 1990, see abstract.	1-16
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
29 July 1991		20 AUG 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		 Abdel A. Mohamed

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Acta Veterinaria (Beograd), Volume 25, No. 4, issued 1975. DURISIC et al., "Antibodies in blood, colostrum and milk sera of sows inoculated with an experimental vaccine of <u>Mycoplasma suis pneumoniae</u> ", pages 189-194, see summary.	1-16
Y,P	US,A, 4,981,684 (Mackenzie et al) 01 January 1991, see example 7.	17-25
Y,P	US,A, 4,985,243 (Faulds et al.) 15 January 1991, see column 4, lines 14-27 and Examples 1-4.	17-25